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INFECTION EXPOSURE, DETECTION AND CAUSES OF DEATH IN PERINATAL MORTALITIES IN POLISH DAIRY HERDS

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ABSTRACT

The objective of this study was to determine the prevalence and types of infections in perinatal mortality (PM) cases from Polish dairy farms and the relevance of the presence of infection to the cause of death. This prospective longitudinal study was carried out on 121 PM and 21 control calves with a gestation of ≥260 days. Six control calves were euthanized and examined using the same protocol as for PM calves. Material was collected over a 20-month period between November 2013 and June 2015. The PM and control calves were collected from 29 and 5 herds, respectively.

Blood samples from calves were tested for antibodies to *Neospora caninum*, glycoprotein B of BoHV-1, BVDV and SBV using ELISAs and *Leptospira hardjo* and *Leptospira pomona* with the microscopic agglutination test. Brain and kidney samples from all PM and six euthanized control calves were tested using real time PCR to detect *Neospora caninum*, pathogenic *Leptospira* spp., BoHV-1 and SBV; brain was examined histopathologically for detection of *N. caninum* cysts. Samples from eight inner organs from all PM and six control calves were cultured aerobically, anaerobically and microaerobically. Ear samples from all PM and control calves were tested for BVDV using an antigen ELISA.

In total, 21.5% of PM calves were infected (antigen and/or antibody-positive) *in utero*; none of the control calves were infected. Direct evidence of infection (culture, Ag-ELISA, PCR, histopathology) was detected in 9.1% of PM calves. Gestation length in infected singletons was shorter than in uninfected singletons (274±8 vs. 279±7 days; $P<0.01$). The odds ratio for diagnosis of infection in single pregnancies ≤275 days was 3.75 (95% CI:1.2-12.1), ($P<0.05$). Infection was the cause of death in 10% of calves. The most common infections detected in these Polish PM calves were parasitic (11.6% of PM cases), viral (7.4%) and bacterial (5%). This study demonstrated that indirect evidence of infection is detected more frequently than direct, coinfection is rare, infection is rarely accompanied by gross lesions and is rarely a cause of death in cases of PM.

Keywords: perinatal mortality; stillborn calves; intrauterine infection; precolostral antibody

1. Introduction

Perinatal mortality (PM) has a detrimental effect on cow health, survival, reproductive performance and milk production [1, 2]. Perinatal mortality may be defined as calf death at full-term (≥260 days), prior to or during, or within 12-48h of parturition [3, 4]. There is wide variation in the incidence of PM by farm [2]. In Poland the mean PM rate was 8.1 and 4.8% in first and later calvings, respectively [5]. Important risk factors for PM include age at first calving [6], breed of dam, breeding method, calving management, feto-maternal health status, length of gestation [7], gestational nutrition, calf sex and sire [4].

The causes of death in PM are multifactorial and include non-infectious and infectious causes. The major causes of bovine PM, based on necropsy studies, are dystocia and anoxia and to a lesser extent, infections and congenital defects [8]. Compared to abortion (birth of a non-independently viable fetus pre-term), infection is a less common cause of death in PM calves accounting for 3 to 12% of cases [8].

Numerous infectious agents have been detected in cases of bovine PM including; *Salmonella* Dublin, *E. coli, Aeromonas, Proteus, Streptococcus, Staphylococcus, Neisseria, Absidia, Acinetobacter* species [9], *Corynebacterium pyogenes, Leptospira* species [9, 10], *Bacillus licheniformis, Mannheimia varigena* [11], *Listeria* spp., *Neospora caninum* [12], *Salmonella Stanley* [13], *Coxiella burnetii* [12,14], *Brucella abortus, Aspergillus* spp., *Campylobacter* spp., *Trichomonas foetus* [15], bovine viral diarrhea virus (BVDV) [17, 18], orthobunyaviruses including the recently emerged Schmallenberg virus (SBV) [16] and bovine herpes virus 1 and 4 (BoHV-1, BoHV-4) [17, 18]. It is recognized that some of these isolates are probably contaminants (e.g. *Proteus*), while others are probably secondary opportunistic infections (e.g. *Streptococcus*). Although there are many reports investigating infection in abortion cases, few focus on involvement of infectious agents in PM cases and in some cases results for aborted and PM calves are combined [18-20].

There is no information about the prevalence of infectious agents in PM calves in Poland. Therefore the objective of this study was to examine PM cases from Polish dairy farms to detect direct and indirect evidence of infections and the relevance of the presence of infection to the cause of death. A secondary objective was to compare findings in PM cases with new-born live calves.

2. Material and methods

The study design was approved by the II Local Ethics Commission in the Wroclaw University of Environmental and Life Sciences (permission no. 23/2012, 58/2014, 60/2014).

2.1. Calves

This prospective study was carried out on 121 perinatal mortalities (PM) and 21 control calves (C). Material was collected over a 20-month period between November 2013 and June 2015. None of the calves received colostrum.

Perinatal mortalities were defined as calves born following a gestation of ≥260 days which died before, during or within 6 hours after birth (a case definition of death within 24 hours was planned but no calves died between 6 and 24 hours after birth). These calves were either Holstein-Friesian (n=113) or Holstein-Friesian crossbreds (Simmental, Jersey, Limousine, Brown Swiss sires; n=8).

Control calf inclusion criteria were: gestation length ≥260 days, singleton calving, and Holstein-Friesian breed. All C calves (6 females and 15 males) were born after assisted calvings (6 calves without and 15 with a calving jack). From this group six male C calves (selected on the basis that owners agreed to sell the newborn male calf) were euthanized between 1.30 and 8.30 hours after birth. They were premedicated with 1 ml (20 mg) xylazine (Sedazin®, Biowet Pulawy) and 1 ml (100 mg) ketamine (Bioketan®, Vetoquinol) IV and

then euthanized with a mixture of pentobarbital and pentobarbital sodium, 160 mg/ml (Morbital®, Biovet Pulawy) IV at a dose of 48-96 mg/kg bw.

2.2. Herds

The study was conducted in 30 herds in the south-west of Poland. Herds were recruited as a convenience sample located within 2.5 hour one-way driving distance from the University. Each farmer was provided with a mobile phone number to call the three veterinarians who collected the material on all days and times (day and night) as soon after calf death as possible. Information on the farm management, herd records and details of the calving associated with the calf were collected at farm visits. These data included information about the herd (number of lactating cows, milk yield in the previous lactation, intercalving period, vaccination program), cow – (date of the last service), and calving (single or twin/triplets). The degree of calving assistance was recorded in seven categories (supplied to the farmers): unobserved, observed but not assisted, normal assisted calving without calving jack, normal assisted calving with calving jack, difficult calving with calving jack, difficult calving without calving jack (assistance by at least three people and/or a veterinarian) and caesarean section.The median herd size was 73 (range 1-1037 cows/herd) and the mean (SD) inter-calving period and previous lactation milk yield was 415 (38) days and 8884 (1442) kg/cow/305 DIM, respectively. Fifteen herds were vaccinated in 2013-2015; against BVD alone (n=7), BoHV-1 alone (n=4) or against BVD and BoHV-1 (n=4). PM and C calves were collected from 29 and 5 herds, respectively. Between 1 and 35 cases of PM and between 2 and 7 cases of C calves were investigated per herd.

2.3. Necropsy and laboratory examinations

Necropsies were performed by the same three veterinarians, at least two of which were present at the same time in the necropsy laboratory at the University of Environmental and Life Sciences, Wroclaw. All carcasses (PM and six C calves) were subjected to systematic external and internal gross examinations and sampling according to the same project-specific protocol.

2.3.1. Samples

Samples from an ear, blood, abomasal contents and eight internal organ tissues (spleen, liver, lung, small intestine, left kidney, left adrenal gland, heart, brain) were collected from 121 PM calves. From C calves ear and blood samples were collected from 21 cases. Abomasal contents and eight internal organs (the same as in PM cases) were collected from 6 euthanized calves. For each calf a sterilized set of surgical instruments was used and during necropsy ethanol and flame sterilization was performed. Abomasal fluid was collected (by abomasocentesis) immediately after opening the abdominal cavity with a sterile needle and syringe.

2.3.1.1. Ear samples

Ear biopsies were collected using an ear notcher and individually tested for bovine virus diarrhoea virus (BVDV) using an antigen ELISA (IDEXX BVDV Ag/Serum Plus Test, Hoofddorp, The Netherlands) by the veterinary laboratory Vetlab LP, Wroclaw, Poland. The result was regarded as negative when the optical density (OD) sample minus negative (S-N) value was $\leq$0.2, suspect >0.2 and $\leq$0.3 and positive when OD >0.3.

2.3.1.2. Blood samples

Blood from a jugular vein was aseptically collected by syringe and immediately dispensed into 5 ml lithium heparinized tubes (MEUS Srl®, Piove di Sacco, Italy, 18648), centrifuged (14 minutes; 1860×g), and plasma samples were aliquoted and frozen at -80^{o}C until analysed. Plasma samples were tested for antibodies to *Neospora caninum*, glycoprotein B of BoHV-1 and BVDV using the IDEXX Neospora Ab Test, IDEXX IBR gB X3 Ab Test and IDEXX BVDV Total Ab Test (Hoofddorp, The Netherlands), respectively, at the veterinary diagnostic laboratory (Weterynaryjna Diagnostyka Laboratoryjna, Gietrzwald, Poland). The sample was classified as seropositive for *Neospora caninum* when the OD S/P was ≥0.5. For the BoHV-1 ELISA, the blocking % was calculated and samples with blocking values <45% were regarded as negative; values between 45–55% were inconclusive and values >55% were regarded as positive. In the BVDV Ab ELISA, samples with S/P OD values <0.2 were classified as negative, 0.2 to <0.3 as inconclusive and ≥0.3 as positive.

Plasma was tested for antibodies to SBV using the Serological ID Screen Schmallenberg Virus Competition Multi-Species ELISA (ID-vet, Grabels, France) at the National Veterinary Research Institute (Puławy, Poland). ELISA S/P values <40% were regarded as negative; values between 40–50% were inconclusive and values >50% were regarded as positive.

Plasma was tested also for antibodies to *Leptospira hardjo* and *Leptospira pomona* using live strains of *L. hardjo* and *L. pomona* in the microscopic agglutination test at the veterinary diagnostic laboratory (Weterynaryjna Diagnostyka Laboratoryjna, Gietrzwald, Poland) according to the Instruction of the Polish Chief Veterinary Officer [21]. Plasma samples were tested at an initial dilution of 1:50. On a microscopic slide, equal volumes of plasma were incubated with one of the serovars in a humid chamber at 29±1° C for 2 hours. The results were evaluated using dark-field microscopy.

Poland has ‘Brucellosis-free’ status according to EU regulations [22], therefore samples were not examined for *Brucella* spp.

2.3.1.3. Culture of abomasal and organs samples

Samples were cultured aerobically at 37° C; for routine culture: Columbia Agar with 5% Sheep Blood (Graso, Poland), MacConkey Agar with crystal violet (Graso, Poland); for *Salmonella* spp. culture: buffered peptone water (Graso, Poland), Rappaport-Vassilidasis broth (Graso, Poland) Agar XLD/SS (Graso, Poland) and for anaerobic and microaerobic culture: Columbia Agar with 5% Sheep Blood (Graso, Poland), Schaedler Broth + wit K3 (Graso, Poland) with Atmospheric generators GENbag anaer (Biomerieux, Marcy-l'Étoile, France) and GENbag microaer (Biomerieux, Marcy-l'Étoile, France). Culture results were considered positive when pure growth of the same bacterium was detected from all eight inner organs and the abomasum.

2.3.1.4. Molecular detection of pathogens

Samples of internal organs were tested using real time PCR to detect *Neospora caninum,* pathogenic *Leptospira* spp., BoHV-1 and SBV. Sections of left midbrain cortex were tested for *Neospora caninum* using the Adiavet™ Neospora Real Time (Adiagene, Saint-Brieuc - France). For SBV detection brain RNA extraction was carried out using TRI reagent and real-time RT-PCR with bovine β-actin internal control [23]. Sections of left kidney were tested using the Adiavet™ Lepto Realtime (Adiagene, Saint-Brieuc - France) in LABOklin GmbH & Co, Bad Kissingen Germany and sections of liver were tested using the Vetmax BHV-1 (Applied Biosystems, Foster City, USA) in the LUFA-Nord West, Institute for Animal Health (Oldenburg, Germany).

In all calves where the plasma was positive to either *L. hardjo* and/or *L. pomona* or *N. caninum* or where *N. caninum* cysts were observed in brain, additional TaqMan real time PCR testing was performed separately in each of four sections of the kidney and brain samples,

respectively, in LABOklin GmbH, Bad Kissingen Germany. Detection of *Leptospira* sp. was carried out according to Stoddard et al., [24] and *N. caninum* according to Pereira et al., [25].

2.3.1.5. Histopathology

The right half of the brain was sliced into 3 parts and fixed with 4% buffered formaldehyde (Chempur, Piekary Śląskie, Poland). Following fixation the brain was dissected into five slabs: 1st cut - optic chiasm, frontal cortex, lower temporal cortex, the front part of lateral ventricle, corpus striatum and olfactory triangle; 2nd cut - in the proximity of gray tuberculum and infundibulum hypophysis, through fronto-parietal cortex, temporal cortex, corpus amygdaloideum, lateral ventricles, III ventricle, thalamus and hypothalamus to the proximal part of hippocampus; 3rd cut -from the oculomotor nerve, through parietal cortex, temporal cortex, rostral lamina, brain stem, body of the corpus callosum and through hippocampus; 4th cut -from the trapezoid body, through the cerebellar vermis, IV ventricle, trapezoid body and pyramid; 5th cut -near the obex, through the medulla oblongata. Brain sections were embedded in paraffin, cut into 4-7µm slabs and stained with H-E (Hematoxylin H3136 Sigma-Aldrich, Eosin Y 230251 Sigma-Aldrich). A diagnosis of *N. caninum* infection was based on the observation of *N. caninum* cysts in any brain section. The size of the cyst and the thickness of the cyst wall were measured with a Zeiss Axioskop 50 microscope using ImageView x86 software.

The criteria used to diagnose infection as a cause of death were: culture of a significant pathogen in pure or nearly pure growth from the foetus, and/or detection of gross/histological lesions of infection in the fetus and/or detection of antibodies to significant pathogens in the fetus where other causes of death were excluded [26]. Cases of co-mortality where infection could have contributed to, but was not the only cause of, death, were excluded.

226 2.4. Statistical analysis

227 The gestation length of singleton PM cases with and without any diagnosis of infection was
228 compared using the student T-test and the odds ratio (OR) for diagnosis of infection in single
229 pregnancies ≤275 days were calculated in STATISTICA 12.5.

230

231 **3. Results**

232

233 3.1. Perinatal mortalities

234 All necropsies of PM calves were performed within 8±3h after calving. The results of
235 pathogen identification from the PM calves are shown in Table 1. Fifty per cent of herds and
236 14.9% of calves showed evidence of exposure to infection (antibody-positive). Direct
237 evidence of infection (culture, Ag-ELISA, PCR, histopathology) was detected in 9.1% of PM
238 calves. In 13 abomasal samples bacteria were cultured; seven cases of *E.coli,* four cases of
239 mixed growth with *E.coli*, one *Staphylococcus* coagulase negative and one *Enterococcus*
240 *faecalis*. In only two of these cases was the same bacterium (*E. coli* and *E. faecalis*) cultured
241 from all other inner organs.
242 In total, 21.5% of calves were infected (antigen and/or antibody-positive) *in utero*. Only two
243 infected calves had gross lesions probably associated with infection; one had intra-uterine
244 growth retardation (IUGR), (7.7 kg, *L. hardjo* Ab-positive, single calf) and one had
245 incomplete ossification of the hard palate (SBV Ab-positive).
246 Coinfection was detected in four calves; one calf was positive for bacterial culture (*E.*
247 *faecalis)* and had *N. caninum* cysts in the brain; one calf was positive for *L.hardjo* 1:400 and
248 *L.pomona* 1:200 Ab; one calf was positive for *N. caninum* cysts in the brain and SBV Ab;
249 and one calf was positive for *N. caninum* cysts in the brain and BVDV Ab (Table 1).

The range in the size of the *N. caninum* cysts found in the brain was 13.9-31 x 15-32.6 µm with a wall thickness of 0.9-2.1 µm. The percentage of calves infected with *N. caninum* is shown in Table 1.

Eight and seven herds with and without infection in stillborn calves, respectively, had been vaccinated against BVD and/or BoHV-1. The degree of calving assistance in calves with and without diagnosed infection is shown in Table 2. Dystocia was recorded in approximately 40 and 50% of calves with, and without, infection, respectively.

Mean ± SD gestation length was numerically longer for singletons (278±7 days) compared to twins/triplets (271±5 days). Gestation length in infected singletons was shorter than in uninfected singletons (274±8 vs. 279±7 days; P<0.01). The OR for diagnosis of infection in single pregnancies ≤ 275 days was 3.75 (95% CI:1.2-12.1), (*P*<0.05).

3.2. Control calves

Necropsies of control calves were performed within 6±3h after calving and within 2h±40 minutes after death. Mean pregnancy length ± SD was 279±4 days. No pathogens were detected by culture (except two abomasal samples in which *E. coli* was isolated) or by PCR from the six euthanized control calves. Pathogen-specific antibodies or BVDV antigen were not detected in any of the 21 control calves.

4. Discussion

The results of this study show that approximately 21% of PM calves in these Polish dairy herds were exposed to pathogens during pregnancy; 15% had pathogen-specific antibodies and 9% had pathogenic antigens detected (some had both). Unlike many routine diagnostic laboratory-based studies of infections in bovine fetuses, where data from aborted and stillborn fetuses are combined, [e.g. 14, 18-20], in the present study aborted fetuses were excluded,

hence the prevalence of antibodies and pathogenic antigens was lower. For example, a Dutch study detected pathogenic antigens in 28% of aborted and stillborn fetuses [14]; three-times higher than the prevalence in the present study. Where aborted and stillborn fetus data are disaggregated, within the same study, detection of pathogenic antigens is always significantly lower in stillborn calves. For example, at the lower end, a Canadian study diagnosed an infectious cause of death in 16% of aborted and in only 3% of stillborn fetuses [12] while at the higher end, a Finnish study detected significant bacterial isolates in 26 and 10% of aborted fetuses and stillborn calves, respectively [11]. The results from the present study (9% antigen detection) are within this range (2-10%). These findings indicate that at term non-infectious causes of death, e.g. dystocia and asphyxia, are more important than infectious causes of death.

The results presented indicate that infection causes stillbirth in a minority of cases, compared to aborted fetuses. Specifically, excluding co-mortality, it is estimated that 10% of calves (12/121), approximately half of all infected calves (12/26), died due to infection. This incidence is within the range described in the literature [8] suggesting that infection causes a similar proportion of stillbirths in Polish calves as internationally. Even one cannot assume all detected infections are a cause of PM, the significantly shortened gestation (by 5 days, after excluding multiple pregnancies) of infected calves indicates that infection probably detrimentally affected fetal maturity and viability at calving. The negative effect of infection on gestation length is supported by the similar gestation length of uninfected PM cases with C calves, in which no infection was diagnosed.

There was a similar distribution of difficult calving (which is considered the most important cause of PM [8]) amongst calves with and without detected infections suggesting no interaction between degree of calving assistance and fetal infection.

In this study 15% of stillborn calves had pathogen-specific antibodies. The synepiteliochorial bovine placenta prevents the passage immunoglobulins from dam to fetus [27]. Although some studies [28, 29] suggested possible leakage of maternal antibodies to the fetus, the possibility of endogenous fetal antibody production could not be ruled out. Hence, the antibodies detected in the stillborn calves are likely to have originated from the fetus; the fetus can produce IgM and IgG antibody by the second trimester [30].

In the present study, the detection of a humoral immune response was more frequent (15%) than direct evidence (9%) of pathogen infection. This apparent discordance could be due to the time lapse between infection and fetal death, sample collection issues or limitations of the diagnostic methods, which are not as sensitive for autolytic samples. The ability to mount an immune response can determine the outcome of infection of the fetus [31]. In the absence of defence mechanisms and/or a highly virulent pathogen, intrauterine infection may result in abortion. The presence of antigens without an antibody response is an indicator of infection, either very early in pregnancy (before fetal immunocompetence), or later in pregnancy when the infection kills the fetus before it seroconverts. The detection of pathogens at term, which infect the fetus early in gestation, requires that the agent or its genetic material persists in the fetus, which is not always the case. Therefore, the detection rate and the role of infectious agents in the aetiology of PM maybe artificially low and underestimated, respectively.

Bacterial infections are not common in PM calves. Murray et al. [32] reported a similar frequency of stomach content positive cultures (for *B. licheniformis*), (2%) in stillborn calves to that in the present study (<2%).. Similarly, Agerholm et al. [33] found a very low incidence of bacterial infection (*Bacillus licheniformis*) in stillborn calves (0.8%). A higher percentage of culture positives was recorded in a Finnish study [11] where a significant bacterial isolation was made in 10% of calves which died within 24 hours of birth.

In the present study pure growth of pathogenic bacteria was detected in less than 2% of PM calves. In one case, *E. coli* was isolated. This is one of the most common bacteria isolated from PM and aborted calves [11, 34]. In another case *Enterococcus faecalis* was isolated. This is an opportunistic pathogen detected as a secondary infection in a case where *N. caninum* was diagnosed. One possible source of this pathogen could be an ascending infection in the reproductive tract. The poor correlation between isolation of bacteria from abomasal samples and internal organs detected here suggests that the former samples may not be representative of systemic organ infection. Abomasal samples are routinely used in veterinary diagnostic laboratories to detect bacteria in aborted and stillborn calves. However, the number of culture-positive calves was small in this study, limiting firm conclusions in this regard.

The detection of low titers to *Leptospira* sp. (1:100-1:200) does not exclude the involvement of these bacteria in PM. Calves experimentally infected during pregnancy with *Leptospira* serovar *hardjo* which had different outcomes (apparently viable, weak, dead) varied in the extent of their immune response (microagglutination titers from undetectable to 1:30 000 in precolostral sera) [35]. However, the absence of *Leptospira* spp. genetic material in real time PCR excludes the likelihood of extensive infection in the present study. The incidence of leptospiral infection in PM calves (3.3%) was much lower than that reported in Northern Ireland (15-26%) [9, 10]. The lower infection rate in those studies was associated with increased use of vaccines against leptospirosis, but in the present study leptospira vaccines were not used in any of the herds.

In this study, BoHV-1 was not detected (directly or indirectly). This may be because BoHV-1 infection is uncommon in PM calves in Poland (there are no published studies with which to compare). While the presence of infection cannot be excluded,IBR virus was not detected in the liver, as reported by Smith et al. [17], where the virus was isolated in many tissues (i.e. spleen, lung, brain, ileum) of PM calves, but not in the liver. The absence of antibody to

BoHV-1 in the present study calves may be because this virus acts immunosuppressively, as in foetuses whose dams were experimentally infected, which mounted no antibody response although virus was detected in all organs (lungs, liver, spleen, kidneys, intestine, mesenteric lymph nodes) [36]. It may be the same as with BoHV-4 infection in bovine foetuses that can be infected *in utero*, but are born seronegative [37]. Additionally, as in almost 30% of herds in the present study cows were vaccinated against BoHV-1, this could partly explain the failure to detect BoHV-1. It has been shown that BoHV-1 challenge of vaccinated cows resulted in the birth of normal calves in which BoHV-1 virus was not isolated [17]. Thus, it is the most probable that IBR infection in PM calves was not present in these Polish dairy herds.

SBV was first detected in cattle in Poland in 2012 [39] and the virus spread very efficiently in the ruminant population with a ten-fold increase in seroprevalence in 2013 [40]. SBV antibodies were detected in three herds in the present study where 5 of the 24 PM calves tested were Ab-positive (21%) and none of the eight C calves from these herds was Ab-positive; in total 4.1% of stillborn calves from 29 herds were seropositive. Given these low herd- and animal-level seroprevalences no clinical significance can be attributed to the presence or absence of antibodies in stillborn or live born calves. The presence of SBV antibodies in calves in the present study without gross malformations (AHS - arthrogryposis, hydranencephaly syndrome) suggests infection occurred after 47 days of gestation when the foetus was immunocompetent [38]. Most fetuses infected after immunocompetence are born alive and seropositive but without lesions indicative of SBV infection [38]. Thus, in the present study the foetuses were exposed to SBV, mounted an immune response, but died due to non-SBV causes and showed none of the pathognomonic pathological lesions of the AHS. These findings are not surprising in a country with endemic seroprevalence to SBV.

N. caninum was the most commonly detected pathogen in these PM calves. The percentage of PM calves with a positive humoral response (4.1%) was similar to that reported by Graham et

al. [41], where 5.5 % of PM or weak calves had an antibody titre ≥1/320 to *N. caninum*. The timing of foetal infection with *N. caninum* determines the outcome of the pregnancy. The occurrence of cell destruction, and therefore disease, depends upon the balance between tachyzoites being able to penetrate and multiply in host cells and the ability of the host to inhibit parasite multiplication [42]. Infection in late pregnancy allows the fetus to suppress infection and pregnancy ends in time for clinically normal calves to be born. The highest rate of detection the *N. caninum* is in the brain. This may be because the parasite has a predilection for the central nervous system [42]. The size of the cysts observed in the present study correspond to *N. caninum* cysts observed in other studies [43] and the wall thickness excludes *Toxoplasma gondii* cysts which have a thinner wall (<0.5µm) [44]. In the present study, each brain sample from each individual calf was dissected in five slabs and was thoroughly examined. This probably enabled a high detection rate. However, all the initial real time PCR *Neospora* results were negative, possibly because only a small amount of tissue (10 mg) was examined. In the second run, when all the positive samples in either serology or histopathology were retested using four separate samples from each brain, one positive case was detected. This implies that the infection rate was low and could have been underestimated if only a single run molecular test protocol was adopted. In the experimental study by Innes et al. [45], *N. caninum* DNA were detected in the brain of most calves after infection of naive cows during mid-gestation, however, DNA extraction was performed on 1 g of tissue.

In the present study, BVDV antibody was detected in 3.3% of PM calves, which indicates that infection occurred in the later stage of pregnancy, after 125 days of gestation [46]. The presence of seropositive calves is indirect evidence of virus exposure in the herd, so infection of BVDV could be present in other calves from the same herd in earlier stages in pregnancy leading to persistently infected (PI) calves, but none of the PM calves were PI. The percentage of PI in newborn calves is low internationally. For example, for every eight seropositive pre-

colostral calves on commercial dairy farms in the USA, one PI was born [47]. So one cannot exclude the possibility that if more PM calves had been tested, PI calves would have been detected as in the study by Smith et al. [9] where BVDV antigen was detected in 0.7% of PM calves.

In four calves (3.3%) coinfections with different pathogens were detected. Dual infection in a single calf is not unexpected [9, 41], but reports of multiple PM calves with coinfections are uncommon. Diagnosis of multiple infections in PM cases, after excluding opportunistic agents, suggests a high environmental infectious challenge in these herds; specifically, with *N. caninum* which was the coinfection in three of the four coinfected calves.

One of the limitations of studies such as this is that the type of infection and the prevalence of pathogens may be herd- and country-specific. Hence, one cannot cite data on infection rates and types in the international literature without reference to the country in which the data were collected. Thus, in countries free from specific infections, e.g. *Brucella abortus* (Poland, Republic of Ireland), Bovine Herpes Virus-I (Sweden, Finland) or pathogenic *Leptospira* spp. (Sweden) [3, 11], those infectious agents will not be detected in PM calves. This does not reduce the importance of reporting the findings, but merely highlights the need to take cognizance of potential geographical factors.

Another limitation of such studies is the variation in sampling protocols. In this study, where all calves were tested, irrespective of gross pathology, the most commonly detected pathogen was *N. caninum*. However, in a Danish study where only tissues with macroscopic pathological lesions were sampled the most common infection in PM calves was BVD virus [33]. These protocol differences may account for a lower infection detection rate in studies where selective sampling is implemented and a relatively higher detection rate in the present study. In future, immune and inflammatory biomarkers may be used to diagnose bovine fetal infections [48].

5. Conclusions

While one fifth of PM calves were infected *in utero*, detection of a humoral response was almost twice as common as detection of a pathogen. However, infection contributed to the cause of death in only 10% of stillborn calves. These findings indicate that while fetal exposure to pathogens is not uncommon during bovine pregnancy, infection at term is uncommon and infection causing stillbirth is even less common. Given that the prevalence and types of infection in PM calves may be country- and herd-specific, extrapolation of any pathogen-specific results beyond the environment in which they were detected should be made with caution. Notwithstanding this caveat, the findings generated in this study from a detailed sampling protocol for multiple causative agents in all calves indicate that infection rates in PM cases may be underestimated in published studies. In Polish herds the most common infections in PM calves, in descending order, were parasitic, viral and bacterial.

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565

566 Table 1. Bacterial, parasitic and viral pathogens detected in 121 cases of perinatal mortality
567 from 29 Polish dairy herds.
568 Table 2. The degree of calving assistance in PM calves with (n=26) and without (n=95)
569 diagnosed infection (antibody and/or antigen) *in utero*.
570

571 Table 1. Bacterial, parasitic and viral pathogens detected in 121 cases of perinatal mortality
572 from 29 Polish dairy herds.

Pathogen type	Pathogen	Detection method	n/N*	Positive		Cumulative infection n (%)
				Calves % (titer)	Herd Code No.	
Bacterium	*Escherichia coli*	Culture	1/121	0.8	14	6 (5.0)
	Enterococcus faecalis	Culture	1/121	0.8	10[1)]	
	Leptospira sp.	Real time PCR (kidney)	0/120	0	-	
	Leptospira hardjo	MAT	4/121	2.5 (n=3; 1:100) 0.8 (n=1; 1:400)	6,3,16,7[2)],	
	Leptospira pomona	MAT	1/121	0.8 (1:200)	7[2)]	
Parasite	*Neospora caninum*	Brain histopathology	9/119	7.6	8, 7, 7, 4, 10[1)], 11, 12[3)], 16, 23[4)]	14 (11.6)
		Real time PCR (brain)	1/120	0.8	1[†]	
		Ab ELISA	5/121	4.1	1[†], 4, 4, 7, 21,	
Virus	BVDV	Ag ELISA (ear) (121)	0/121	0	-	9 (7.4)
		Ab ELISA	4/121	3.3	7, 12, 18, 23[4)]	
	BoHV-1	Real time PCR (liver)	0/120	0	-	
		Ab ELISA	0/121	0	-	
	SBV	Real-time RT-PCR (brain)	0/120	0	-	
		Ab ELISA	5/121	4.1	3, 12[3)],12, 13,13,	

573 *number of positive calves to the number of all calves tested; MAT- microscopic
574 agglutination test, Ab-antibody, BVDV- Bovine viral diarrhea virus, BoHV-1 Bovine
575 herpesvirus 1, SBV- Schmallenberg virus
576 [1-4)] four calves with coinfection; the same superscript in different rows indicates the different
577 coinfections present in the same calf, e.g. calf[1] was coinfected with *E. faecalis* and *N.*
578 *caninum*.
579 [†]infection by *N. caninum* was diagnosed in the same calf by different methods (this is not
580 coinfection)
581

582 Table 2. The degree of calving assistance in PM calves with (n=26) and without (n=95)
583 diagnosed infection (antibody and/or antigen) *in utero*.

Degree of calving assistance	**Calves with diagnosed infection (%)**	**Calves without diagnosed infection (%)**
Unobserved	11.5	4.2
Observed, not assisted	0	3.2
Assisted without calving jack	34.6	14.7
Assisted with calving jack	15.4	30.5
Difficult calving without calving jack	7.7	8.4
Difficult calving with calving jack	30.8	35.8
Caesarean section	0	3.2

584

- Exposure to pathogens during pregnancy as detected in approximately 21% of perinatal mortality calves
- A humoral immune response was detected approximately twice as frequently as direct evidence of pathogen infection.
- Gestation length of infected singletons was significantly shorter than that of uninfected singletons.
- The most common infections in perinatal mortality calves, in descending order, were parasitical, viral and bacterial.